



## Cilostazol inhibits plasmacytoid dendritic cell activation and antigen presentation

Fei SUN<sup>1</sup>, Zhao YIN<sup>1</sup>, Hai-Sheng YU<sup>2</sup>, Quan-Xing SHI<sup>1</sup>, Bei ZHAO<sup>1</sup>, Li-Guo ZHANG<sup>2</sup>, Shou-Li WANG<sup>1</sup>

<sup>1</sup>Department of Cardiology, The 306<sup>th</sup> Hospital of PLA, No. 9 Anxiangbeili Street, Beijing, China

<sup>2</sup>CAS Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing China

### Abstract

**Background** Cilostazol, an anti-platelet drug for treating coronary heart disease, has been reported to modulate immune cell functions. Plasmacytoid dendritic cells (pDCs) have been found to participate in the progression of atherosclerosis mainly through interferon  $\alpha$  (IFN- $\alpha$ ) production. Whether cilostazol influences pDCs activation is still not clear. In this study, we aimed to investigate the effects of cilostazol on cell activation and antigen presentation of pDCs *in vitro* in this study. **Methods** Peripheral blood mononuclear cells isolated by Ficoll centrifugation and pDCs sorted by flow cytometry were used in this study. After pretreated with cilostazol for 2 h, cells were stimulated with CpG-A, R848 or virus for 6 h or 20 h, or stimulated with CpG-B for 48 h and then co-cultured with naïve T cell for five days. Cytokines in supernatant and intracellular cytokines were analyzed by ELISA or flow cytometry respectively. **Results** Our data indicated that cilostazol could inhibit IFN- $\alpha$  and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production from pDCs in a dose-dependent manner. In addition, the ability of priming naïve T cells of pDCs was also impaired by cilostazol. The inhibitory effect was not due to cell killing since the viability of pDCs did not change upon cilostazol treatment. **Conclusion** Cilostazol inhibits pDCs cell activation and antigen presentation *in vitro*, which may explain how cilostazol protects against atherosclerosis.

*J Geriatr Cardiol* 2015; 12: 388–393. doi:10.11909/j.issn.1671-5411.2015.04.011

**Keywords:** Antigen presentation; Cilostazol; Interferon  $\alpha$ ; Plasmacytoid dendritic cell; Tumor necrosis factor  $\alpha$

### 1 Introduction

Cilostazol is widely used for anti-platelet treatment in patients with coronary artery disease, especially after percutaneous coronary intervention (PCI). It is reported that the addition of cilostazol to aspirin and clopidogrel dual anti-platelet therapy is more effective in preventing restenosis and avoiding target-vessel revascularization after PCI.<sup>[1]</sup> However, the exact mechanism underlining this effect is still not clearly understood. Previously, we proved that cilostazol suppresses the differentiation of Th1 and Th17 cells and up-regulates regulatory T cells.<sup>[2]</sup> Other researchers have demonstrated that cilostazol can also down-regulate C-C chemokine receptor type 2 (CCR2) gene expression, so as to reduce monocyte chemoattractant protein-1 (MCP-1)-induced chemotaxis and adhesion of monocytes.<sup>[3]</sup> Moreover,

cilostazol inhibits atherosclerosis by reducing the production of superoxide and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ).<sup>[4]</sup> Besides, it has been recently discovered that cilostazol suppresses TLR2-mediated IL-23 production from macrophage.<sup>[5]</sup> Taken together, the effect of cilostazol on the immune system is of consideration during the clinical treatment.

It is widely accepted that immune cells and cytokines play a vital role during the development of atherosclerosis. Plasmacytoid dendritic cell (pDC) is a special subset of DCs characterized by their high level of IFN- $\alpha$  production upon viral infection or nucleic acid TLR lig and stimulation.<sup>[6]</sup> It is demonstrated that intraperitoneal injection of interferon  $\alpha$  (IFN- $\alpha$ ) accelerated atherosclerosis in low density lipoprotein (LDL) receptor-deficient mouse model fed with western type diet.<sup>[7]</sup> Besides, pDCs in atherosclerotic plaque could induce smooth muscle cells apoptosis through IFN- $\alpha$ .<sup>[8]</sup> In another study, injection of peptide/DNA complex, which triggered pDCs activation via TLR9 to induce IFN- $\alpha$  production, promoted an enhanced atherosclerotic lesion formation in Apoe<sup>-/-</sup> mice. Furthermore, the exacerbation of atherosclerosis was adverted when plasmacytoid dendritic cell Ag-1 (PDCA1) antibody was added to deplete pDCs.<sup>[9]</sup>

Since cilostazol can influence T cells, monocytes and

**Correspondence to:** Shou-Li WANG, MD, Department of Cardiology, The 306<sup>th</sup> Hospital of PLA, No. 9 Anxiangbeili Street, Beijing, China. E-mail: wangsl.63@126.com

**Telephone:** +86-10-66356125

**Fax:** +86-10-66356125

**Received:** November 13, 2014

**Revised:** April 27, 2015

**Accepted:** April 30, 2015

**Published online:** June 3, 2015

macrophages,<sup>[2,3,5]</sup> in this report, we investigated the effect of cilostazol on TLR ligand and virus-induced activation and antigen presentation capacity of pDCs.

## 2 Methods

### 2.1 Isolation of peripheral blood mononuclear cells and pDCs

The study protocol was approved by the Institutional Ethics Committee of 306<sup>th</sup> Hospital of PLA. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll centrifugation. Monocytes, NK cells, T cells and B cells were depleted using a mixture of anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56 mAbs (Biolegend, San Diego, CA, USA) with magnetic beads coated with goat anti-mouse IgG (MiltenyiBiotec, San Diego, CA, USA). The lineage (CD3, CD14, CD16, CD19, CD20) depleted PBMC were stained with HLA-DR (APC-Cy7), CD123 (BV421), CD11c (APC), and HLA-DR<sup>+</sup>CD123<sup>+</sup>CD11c<sup>-</sup>Lin<sup>-</sup>pDCs were sorted with BD FACSARIAIII (BD Biosciences, Franklin Lake, NJ, USA), and the purity of pDCs was over 99%.

### 2.2 Drugs and cell culture

Cilostazol was purchased from Sigma, and dissolve in DMSO at the concentration of  $8 \times 10^4$   $\mu\text{mol/L}$  for storage. PBMCs or purified pDCs, cultured in RPMI 1640 (Hyclone, Logan, UT, USA) containing 10% FCS at  $2 \times 10^5$  cells or  $1 \times 10^4$  per 200  $\mu\text{L}$  in round-bottomed 96-well culture plates, were pretreated with cilostazol DMSO as vehicle for 2 h before stimulated with 2  $\mu\text{mol/L}$  CpG-A (Invivogen, San Diego, CA, USA), 2  $\mu\text{g/mL}$  R848 or 5 MOI HSV. After 20 h of culture, cells and supernatants were harvested for further analysis. The level of IFN- $\alpha$  and TNF- $\alpha$  in supernatant was assessed by ELISA (Mabtech, Nacka Strand, Sweden) or Flow Cytomix (eBioscience, San Diego, CA, USA). Cell viability was determined by Guava Viacount (Guava easyCyte, EMD Millipore, Billerica, MA, USA) and Trypan Blue staining.

### 2.3 Intracellular cytokine analyses

PBMCs were stimulated as indicated for 6 h, and BFA (Biolegend, 1: 1000 dilution) was added in the culture for the last 3 h. Cells were harvested, fixed and permeabilized by using the Cytofix/Cytoperm kit (BD Biosciences). Briefly, cells were first fixed in fixation/permeabilization solution for 20 min at 4°C. Following two washing steps with the permeabilization/washing solution, cells were stained with optimal concentrations of fluorochrome-conjugated antibodies against IFN- $\alpha$  or TNF- $\alpha$  in the permeabi-

lization/washing solution for 30 min at 4°C. After washing three times, cells were suspended in PBS containing 1% fetal calf serum and acquired on LSRFortessa (BD Biosciences) and analyzed by Summit.

### 2.4 T cell proliferation assay

Human CD4<sup>+</sup>CD45RA<sup>+</sup> naïve T cells were purified from PBMC by negative selection with magnetic beads (Miltenyi Biotech, Germany). After 2 h of incubation with cilostazol, pDCs (from 156 to 5,000) were stimulated with 0.2  $\mu\text{mol/L}$  CpG-B (TAKALA, Otsu, Shiga, Japan) for 48 h, then cultured with  $5 \times 10^4$  naïve T cells freshly isolated from PBMCs (naïve CD4<sup>+</sup> T Cell Isolation Kit, MiltenyiBiotec) in round-bottomed 96-well culture plates in RPMI 1640 containing 10% FCS. After five days of pDC-T cell co-cultures, cells were pulsed with 1 mCi [<sup>3</sup>H] thymidine for 18 h before harvesting and counting. Tests were carried out in duplicate and standard deviations were indicated with bars.

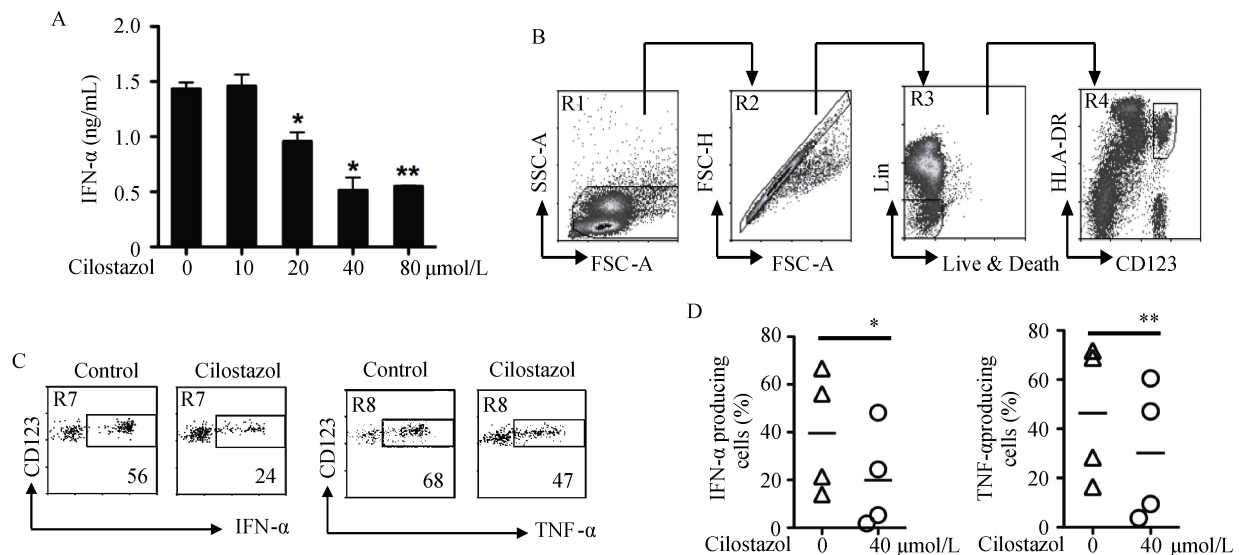
### 2.5 Statistical analysis

GraphPad Prism software (Prism 5 for Mac OS X) was used for statistical analysis. All samples were performed in at least duplicate, with each experiment repeated at least two times. Data are shown as means  $\pm$  SE. Differences between groups were determined using an unpaired or paired, two-tailed, Student's *t*-test.  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  were considered statistically significant.

## 3 Results

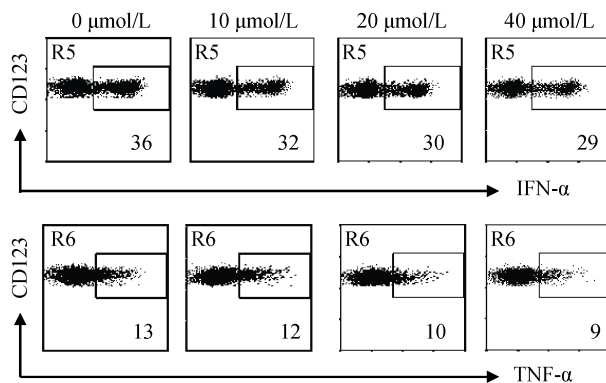
### 3.1 Cilostazol inhibited CpG-A and R848-induced production of IFN- $\alpha$ and TNF- $\alpha$ from PBMCs

The production of IFN- $\alpha$ , which is the most notable characteristic of pDCs, has been proved to promote the progression of atherosclerosis. So, we first determined whether cilostazol could influence IFN- $\alpha$  production. PBMCs were pretreated with cilostazol or DMSO for 2 h before adding 2  $\mu\text{mol/L}$  CpG-A for 20 h. The level of IFN- $\alpha$  in the supernatant was detected by ELISA. According to our results, cilostazol could significantly reduce the production of IFN- $\alpha$  in a dose-dependent manner (Figure 1A), and 40  $\mu\text{mol/L}$  cilostazol had an obvious effect. We further examined the intracellular cytokine level by flow cytometry analysis, and the results were consistent with ELISA data. The gating strategy was shown in Figure 1B. Notably, IFN- $\alpha$  production in cilostazol group was decreased to 38.3% compared to the control group ( $P = 0.019$ , Figure 1C, D). Moreover, we found that the TNF- $\alpha$  production was also inhibited by cilostazol, and reduced to 52.1% on average (Figure 1C, D).



**Figure 1. Cilostazol inhibits CpG-A-induced IFN- $\alpha$  and TNF- $\alpha$  production from PBMCs.** (A): The reduction of IFN- $\alpha$  in supernatant.  $2 \times 10^5$  PBMCs in each well of 96-well plates were pretreated with cilostazol at indicated doses for 2 h before stimulated by CpG-A for 20 h. IFN- $\alpha$  level in supernatant was measured by ELISA. (B): The gating strategy of flow cytometry. (C): The inhibition of cilostazol on intracellular IFN- $\alpha$  and TNF- $\alpha$  production.  $2 \times 10^5$  PBMCs in each well of 96-well plates were pretreated with 40  $\mu\text{mol/L}$  cilostazol for 2 h before stimulated by CpG-A for 6 h and BFA was added for the last 3 h. The amount of intracellular IFN- $\alpha$  and TNF- $\alpha$  was tested by flow cytometry. Data were representative of four independent experiments. (D): Statistical data of Figure 1C. In particular, paired two-tail *t*-test was used to analyze the differences between each donor in different groups. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. control group. CD: cluster of differentiation; FSC: forward scatter; HLA-DR: human leucocyte antigen-D region; IFN- $\alpha$ : interferon- $\alpha$ ; Lin: linear amplification; PBMCs: peripheral blood mononuclear cells; SSC: side scatter; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .

In addition, we also tested the effect of cilostazol on TLR7 ligand and R848 induced inflammation. Similar to CpG-A, both the production of IFN- $\alpha$  and TNF- $\alpha$  induced by R848 was repressed by cilostazol (Figure 2). These data



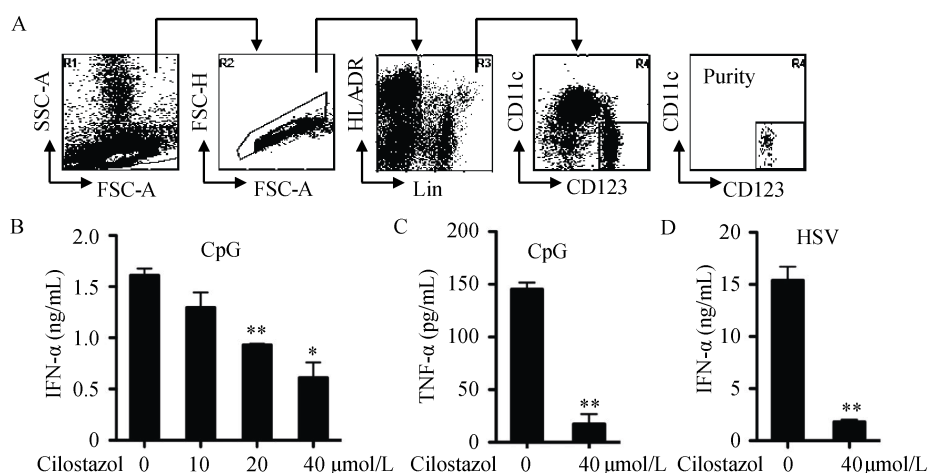
**Figure 2. R848-induced IFN- $\alpha$  and TNF- $\alpha$  production are also decreased by cilostazol in a dose-dependent manner.** PBMCs were pretreated with cilostazol at indicated concentrations for 2 h before stimulated with 2  $\mu\text{g/mL}$  R848 for 6 h and BFA was added for the last 3 h. Intracellular IFN- $\alpha$  and TNF- $\alpha$  was examined by flow cytometry. Data were representative of two independent experiments. CD: cluster of differentiation; IFN- $\alpha$ : interferon- $\alpha$ ; PBMCs: peripheral blood mononuclear cells; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .

of intracellular staining proved that cilostazol influences the protein production of cytokine.

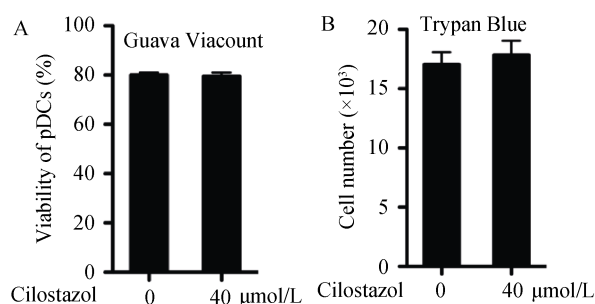
### 3.2 Cilostazol directly suppressed CpG-A and virus induced-cytokine production from purified pDCs

Since there are still several other kinds of lymphocytes in PBMCs, especially T cells, and based on our previous results, cilostazol can also influence the differentiation and function of T cells. So it is still not clear whether the inhibition of cilostazol on pDCs is dependent on other cells or not. To eliminate interaction of other cells with pDCs, we isolated pDCs from PBMCs by FACS to over 99% purity (Figure 3A). Purified pDCs were treated with cilostazol and CpG-A as before. After 20 h, we tested the levels of IFN- $\alpha$  and TNF- $\alpha$  in the supernatant. We found that cilostazol significantly inhibited the generation of IFN- $\alpha$  (Figure 3D) and TNF- $\alpha$  (Figure 3C).

Anti-virus infection is a main function of pDCs, and virus infection, to some extent, contributes to pathogenesis of atherosclerosis.<sup>[10,11]</sup> To fully understand the inhibition of cilostazol on IFN- $\alpha$ , we further stimulated enriched pDCs with two kinds of virus, herpes simplex virus (HSV) and influenza virus (PR8). Consistent with previous results, cilostazol dampened both HSV and PR8-induced IFN- $\alpha$  production (data not shown). To confirm this result, we next



**Figure 3. Cilostazol inhibits CpG-A and virus-induced cytokine production.** (A): The gating strategy and purity of pDCs. (B): Cilostazol inhibited CpG-A-induced IFN- $\alpha$  production from pDCs. Purified pDCs were pretreated with cilostazol at indicated concentrations for 2 h before stimulated by CpG-A for 20 h. The supernatant was used to measure IFN- $\alpha$  by ELISA. (C): Cilostazol inhibited CpG-A-induced TNF- $\alpha$  production from pDCs. Samples were randomly chosen from three independent experiments in Figure 3B, and the levels of TNF- $\alpha$  were determined by FlowCytomix™. (D): Cilostazol directly reduced HSV-induced IFN- $\alpha$  production from pDCs. pDCs were treated with cilostazol and HSV (5 MOI) as in Figure 3B, and the levels of IFN- $\alpha$  were tested by ELISA. One representative of two independent experiments is shown. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. control group. CD: cluster of differentiation; FSC: forward scatter; HLA-DR: human leucocyte antigen-D region; HSV: herpes simplex virus; IFN- $\alpha$ : interferon- $\alpha$ ; Lin: linear amplification; pDCs: plasmacytoid dendritic cells; SSC: side scatter; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .



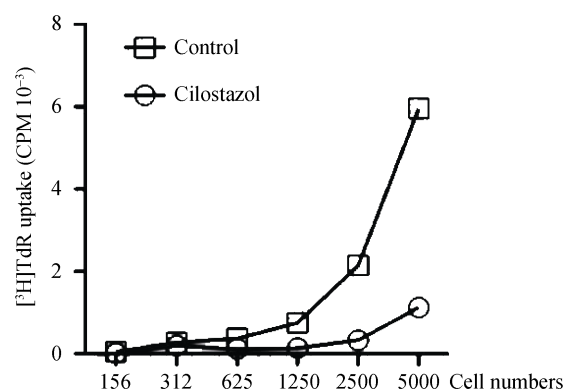
**Figure 4. The viability of pDC does not change upon cilostazol treatment.**  $3 \times 10^4$  pDCs were pretreated with or without cilostazol (40  $\mu$ mol/L) for 2 h, before stimulated by 2  $\mu$ mol/L CpG-A for 8 h. Cell viability was determined by Guava Viacount (A) and Trypan Blue staining (B). Data were representative of two independent experiments. Guava: guava easyCyte; pDC: plasmacytoid dendritic cell.

stimulated purified pDCs with HSV. Cilostazol could also inhibit HSV stimulated IFN- $\alpha$  production ( $P = 0.0092$ , Figure 3D). Stimulated pDCs were counted by Guava Viacount (Figure 4A) and Trypan blue staining (Figure 4D). As shown in Figure 4, the viability of pDCs was not affected by cilostazol, which meant the effect of cilostazol was not due to cell killing.

### 3.3 Cilostazol inhibited pDCs to prime naïve T cells

Antigen presentation is an important function of pDCs. To analyze whether cilostazol could affect pDCs on priming

T cells, pDCs ranging from 156 to 5000 were pretreated with or without 40  $\mu$ mol/L cilostazol for 2 h. Then cells were treated with CpG-B for 48 h before co-cultured with naïve T cells for 5 days. We found that the proliferation of T cells was significantly suppressed with cilostazol treated pDCs (Figure 5), which indicated that antigen presentation of pDCs to T cell were dampened by cilostazol.



**Figure 5. Cilostazol inhibits pDCs to prime naïve T cells.** pDCs were pretreated with cilostazol (40  $\mu$ mol/L) or DMSO for 2 h before stimulated with CpG-B for 48 h, then co-cultured with fresh naïve T cells for 5 d, and pulsed with 0.5  $\mu$ Ci of [ $^3$ H]-TdR (2 Ci/mmol) for 18 h. Cells were harvested onto glass fiber filters and counted for radioactivity. The result was representative of two independent experiments. CPM: counts per minute; pDCs: plasmacytoid dendritic cells; [ $^3$ H]TdR:  $^3$ H-thymidine.

## 4 Discussion

The generation of cytokine, especially IFN- $\alpha$ , is the main characteristic of pDCs, which takes part in the progression of atherosclerosis. As we observed, cilostazol could directly inhibit CpG-A-induced cytokine production, which may explain why the addition of cilostazol to aspirin and clopidogrel showed better clinical benefit. Interestingly, it has also been demonstrated that influenza virus can directly infect and reside in atherosclerotic arteries to promote atherosclerosis,<sup>[10]</sup> so the treatment of cilostazol may partly prevent the progression induced by virus. It has been confirmed that effective T cells promote atherosclerosis through various ways.<sup>[12]</sup> In the present study, we firstly demonstrated cilostazol could inhibit the proliferation of antigen-specific T cells by down-regulating the antigen presentation function of pDCs, so this might be another way to explain how cilostazol protect against atherosclerosis. Taken together, we reported for the first time that cilostazol could suppress pDCs activation which would help us understand the mechanism how cilostazol prevents atherosclerosis.

It is essential to study how cilostazol affects the function of pDCs in patients. However, cilostazol is mostly used in patients after PCI who have taken statins, aspirin or traditional Chinese medicines before admitted to the hospital. So it is difficult to exclude the interference of other drugs. Moreover, even at the peak plasma concentration (2  $\mu$ mol/L), cilostazol only has minimum effect on human pDCs (our unpublished data). Cilostazol would preferentially accumulate at higher concentration in intestines tissue after oral administration and affect pDCs in the digestive tract. However, information on concentrations of cilostazol and studies of the effects of cilostazol on pDCs in intestines tissue are mainly restricted. The effect of cilostazol on pDCs in intestines tissue needs further investigation.

Along with previous results, cilostazol has a very board influence on immune system. It has already caused much attention for more than 20 years that drugs used in cardiovascular diseases can affect immune system.<sup>[13]</sup> In recent years, more and more papers have proposed or verified cardiovascular drugs could affect immune system in which statins is the most noticeable.<sup>[14]</sup> Some researchers have regarded statins as anti-inflammation agents,<sup>[15]</sup> and used them to suppress inflammation and atherosclerosis in the mouse model,<sup>[16]</sup> or even have tried to take statins as a treatment option for immune disorders such as multiple sclerosis.<sup>[17]</sup> Above all, it is noteworthy that many different kinds of common cardiovascular drugs can affect immune

cells during therapeutic process, and we will further clarify how cilostazol influences pDCs in treated patients or animal models.

## Acknowledgement

The authors declare no conflicts of interest.

## References

- 1 Jennings DL, Kalus JS. Addition of cilostazol to aspirin and a thienopyridine for prevention of restenosis after coronary artery stenting: a meta-analysis. *J Clin Pharmacol* 2010; 50: 415–421.
- 2 Wang S, Yan C, Xu H, *et al.* Suppression of encephalitogenic T-cell responses by cilostazol is associated with upregulation of regulatory T cells. *Neuroreport* 2010; 21: 629–635.
- 3 Chuang SY, Yang SH, Pang JH. Cilostazol reduces MCP-1-induced chemotaxis and adhesion of THP-1 monocytes by inhibiting CCR2 gene expression. *Biochem Biophys Res Commun* 2011; 411: 402–408.
- 4 Lee JH, Oh GT, Park SY, *et al.* Cilostazol reduces atherosclerosis by inhibition of superoxide and tumor necrosis factor- $\alpha$  formation in low-density lipoprotein receptor-null mice fed high cholesterol. *J Pharmacol Exp Ther* 2005; 313: 502–509.
- 5 Park SY, Lee SW, Lee WS, *et al.* RhoA/ROCK-dependent pathway is required for TLR2-mediated IL-23 production in human synovial macrophages: suppression by cilostazol. *Biochem Pharmacol* 2013; 86: 1320–1327.
- 6 Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 2005; 23: 275–306.
- 7 Levy Z, Rachmani R, Trestman S, *et al.* Low-dose interferon- $\alpha$  accelerates atherosclerosis in an LDL receptor-deficient mouse model. *Eur J Intern Med* 2003; 14: 479–483.
- 8 Niessner A, Sato K, Chaikof EL, *et al.* Pathogen-sensing plasmacytoid dendritic cells stimulate cytotoxic T-cell function in the atherosclerotic plaque through interferon- $\alpha$ . *Circulation* 2006; 114: 2482–2489.
- 9 Doring Y, Manthey HD, Drechsler M, *et al.* Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation* 2012; 125: 1673–1683.
- 10 Haidari M, Wyde PR, Litovsky S, *et al.* Influenza virus directly infects, inflames, and resides in the arteries of atherosclerotic and normal mice. *Atherosclerosis* 2010; 208: 90–96.
- 11 Pedicino D, Giglio AF, Galiffa VA, *et al.* Infections, immunity and atherosclerosis: pathogenic mechanisms and unsolved questions. *Int J Cardiol* 2013; 166: 572–583.
- 12 Lahoute C, Herbin O, Mallat Z, *et al.* Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets.

- Nat Rev Cardiol* 2011; 8: 348–358.
- 13 Maisel AS. Beneficial effects of metoprolol treatment in congestive heart failure. Reversal of sympathetic-induced alterations of immunologic function. *Circulation* 1994; 90: 1774–1780.
  - 14 Montecucco F, Mach F. Update on statin-mediated anti-inflammatory activities in atherosclerosis. *Semin Immunopathol* 2009; 31: 127–142.
  - 15 Schonbeck U, Libby P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* 2004; 109: II18–26.
  - 16 Liu M, Yu Y, Jiang H, *et al.* Simvastatin suppresses vascular inflammation and atherosclerosis in ApoE(-/-) mice by downregulating the HMGB1-RAGE axis. *Acta Pharmacol Sin* 2013; 34: 830–836.
  - 17 Neuhaus O, Stuve O, Zamvil SS, *et al.* Are statins a treatment option for multiple sclerosis? *Lancet Neurol* 2004; 3: 369–371.